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Limited heterogeneity of rearranged T-cell receptor Vα transcripts in brains of multiple sclerosis patients

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THE identification of activated T cells in the brain of individuals with multiple sclerosis (MS) indicates that these cells are critical in the pathogenesis of this disease. In an attempt to elucidate the nature of the lymphocytic infiltration, we used the polymerase chain reaction to amplify T-cell antigen receptor (TCR) Vα sequences from transcripts derived from MS brain lesions. In each of three MS brains, only two to four rearranged TCR Vα transcripts were detected. No Vα transcripts could be found in control brains. Sequence analysis of transcripts encoded by the Vα 12.1 region showed rearrangements to a limited number of Jα region segments. These results imply that TCR Vα gene expression in MS brain lesions is restricted.

Multiple sclerosis is an inflammatory disease of the central nervous system (CNS), characterized by myelin destruction[1-3]. In the brain, there is an accumulation of macrophages, plasma cells, major histocompatibility complex (MHC) class II positive antigen-presenting cells and activated cytokine-secreting T cells[4-8]. Several lines of evidence indicate that T lymphocytes migrate from the peripheral blood to the CNS compartment and participate directly in the formation of brain lesions[9-11]. There is also evidence of oligoclonality in T lymphocytes within the cerebrospinal fluid of MS patients[12]. In addition, TCR Vα and Vβ genes have been shown to contribute to the genetic control of susceptibility to this disease[13-15].

To examine the expression of TCR genes at the site of disease, messenger RNA isolated from demyelinating brain plaques from three MS patients with chronic progressive disease, and from three control brains (non-MS) was used to synthesize complementary DNA. These cDNAs were then subjected to enzymatic gene amplification by the polymerase chain reaction (PCR) method[16,17] using specific TCR primers. The results of such an amplification using primers for the TCR Vα 12.1 family

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TABLE 1 T-cell receptor α-primers

Primer	Clone	Sequence	Family members
Vα 1	HAP 10	5'-CTGAGGTGCAACTACTCA-3'	1.1, 1.2, 1.3
Vα 2	HAP 26	5'-GTGTTCCCAGAGGGAGCCATTGCC-3'	2.1, 2.2
Vα 3	HAP 05	5'-GGTGAACAGTCAACAGGGAGA-3'	3.1
Vα 4	HAP 08	5'-ACAAGCATTACTGTACTCCTA-3'	4.1
Vα 5	HAP 35	5'-GGCCCTGAACATTCAGGA-3'	5.1
Vα 6	HAP 01	5'-GTCACTTTCTAGCCTGCTGA-3'	6.1
Vα 7	HAP 21	5'-AGGAGCCATTGTCCAGATAAA-3'	7.1, 7.2
Vα 8	HAP 41	5'-GGAGAGAATGTGGAGCAGCATC-3'	8.1, 8.2
Vα 9	HAP 36	5'-ATCTCAGTGCTTGTGATAATA-3'	9.1
Vα 10	HAP 58	5'-ACCCAGCTGGTGGAGCAGAGCCCT-3'	10.1
Vα 11	HAP 02	5'-AGAAAGCAAGGACCAAGTGTT-3'	11.1
Vα 12	PGA 5	5'-CAGAAGGTAACTCAAGCGCAGACT-3'	12.1
Vα 13	AB 11	5'-GCTTATGAGAACACTGCGT-3'	13.1
Vα.14	AB 21	5'-GCAGCTTCCCTTCCAGCAAT-3'	14.1
Vα 15	AC 24	5'-AGAACCTGACTGCCCAGGAA-3'	15.1
Vα 16	AE 212	5'-CATCTCCATGGACTCATATGA-3'	16.1
Vα 17	AF 211	5'-GACTATACTAACAGCATGT-3'	17.1
Vα 18	AC 9	5'-TGTCAGGCAATGACAAGG-3'	18.1
Cα	PGA 5	5'-AATAGGTCGAGACACTTGTCACTGGA-3'	Cα

The size of amplified products using 5' Vα and 3' Cα primers ranged from about 320 to 410 base pairs.

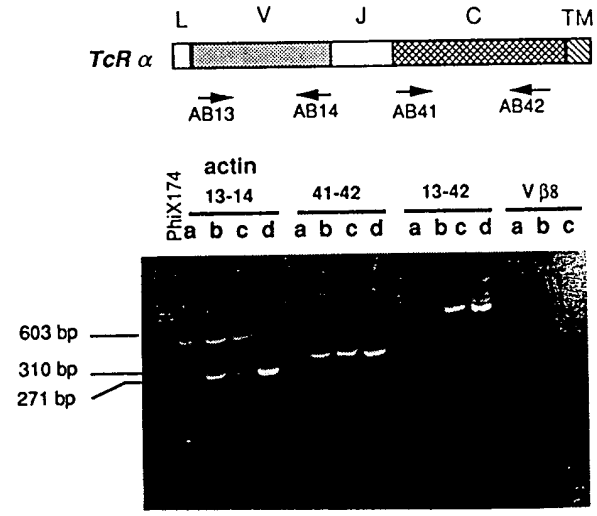

FIG. 1 Brain TCR amplification of MS patient 1. Lanes *a*: control brain cDNA; *b*, MS parietal region brain cDNA; *c*, MS occipital region brain cDNA; *d*, PGA5, a full-length TCR α cDNA[18]. Complementary DNA (2 μl) was combined in a 100-μl reaction volume, with 1 unit of DNA *Taq* polymerase (Perkin Elmer-Cetus), 10 μl 10 × reaction buffer, 50 μM each of deoxynucleoside triphosphates, and 1 μM of each primer. The PCR profile used was: denaturation 95 °C for 60 s, annealing 45 °C for 60 s and extension 72 °C for 60 s, for 35 cycles on a DNA Thermal Cycler (Perkin Elmer-Cetus). One tenth of each sample was independently run in a 4% Nusieve agarose gel (Fmc) and an appropriate size fraction was excised from the gel. The agarose piece was frozen and thawed 3 times, and 2 μl supernatant directly reamplified with the same primers for an additional 25 cycles. A 500-bp actin fragment was successfully amplified from brain cDNA (lanes a-c), but not from the PGA5 control (lane d) using the following primers: 5'-ACGAAGACGGACCACCGCCCTCG-3', 5'-CACGTTGTGGGTGACGCCGTC-3'). Vα and Cα transcripts were amplified from both MS brain cDNA and PGA 5 templates, but not from the control MS brain cDNA with primers AB 13-14 (5'-CAGAAGGTAACTGCAGCGCAGACT-3', 5'-TTGGGGATCCAGAGCACAGAAGTATACTGC-3'), which include *Pst*I and *Bam*HI restriction sites and define a 286-bp fragment of the Vα 12.1 region gene; and AB 41-42 (5'-CAGAACCCTGACCCTGCCGTGTAC-3', 5'-GTGTCCACAGTTTAGGTTCGTATCTGT-3', which includes a *Sal*I site and defines a 340-bp fragment of the Cα region transcript) respectively. Note that rearranged TCRα sequences could be amplified from cDNA of the MS brain prepared from the occipital region (lane *c*) using the Vα 12.1 primer AB 13 and Cα primer AB 42.

TABLE 2 T-cell receptor Vα expression in brain plaques of multiple sclerosis patients

	Vα 1	Vα 2	Vα 3	Vα 4	Vα 5	Vα 6	Vα 7	Vα 8	Vα 9	Vα 10	Vα 11	Vα 12	Vα 13	Vα 14	Vα 15	Vα 16	Vα 17	Vα 18	Vβ 8	Actin
Experiment no. 1																				
MS Br1	383	4,640	760	520	240	850	826	1,566	450	*45,860*	5,430	*36,380*	3,618	367	280	289	226	442	170	*104,450*
MS Br2	140	824	523	310	830	415	660	*23,200*	1,750	*29,630*	623	*49,125*	456	220	317	*12,460*	3,572	338	280	*79,120*
MS Br3	638	313	276	410	817	1,520	210	*15,860*	*16,310*	*21,200*	838	2,050	302	225	462	3,633	482	470	630	*58,358*
C Br4	235	1,100	135	115	286	7,300	427	960	1,036	317	560	726	485	278	466	630	545	830	900	*65,996*
C Br5	580	875	180	490	110	846	160	324	780	120	344	138	762	755	876	860	715	570	860	*66,393*
C Br6	137	290	133	530	836	640	910	110	140	350	670	1,030	1,095	2,000	437	775	240	330	710	*139,337*
Experiment no. 2																				
MS Br1	1,650	3,956	1,450	790	547	545	1,170	343	1,856	*32,870*	513	*12,978*	866	868	3,190	280	1,048	1,127	440	*38,593*
MS Br2	967	340	1,419	1,575	3,866	2,837	1,848	*13,373*	2,974	*17,337*	1,550	*33,020*	1,487	1,072	3,148	*17,968*	1,446	980	1,338	*32,460*
MS Br3	666	726	1,198	790	1,769	258	576	*35,270*	*18,990*	*19,138*	948	2,690	587	880	815	945	946	1,570	630	*22,415*
C Br4	1,507	660	1,740	1,790	553	706	4,540	4,410	1,333	584	919	765	860	206	590	713	2,748	526	864	*31,285*
C Br5	896	1,670	2,370	5,000	2,826	418	862	8,175	2,048	1,307	1,734	836	737	1,040	2,097	2,925	1,025	5,276	4,478	*33,018*
C Br6	883	1,727	716	865	610	1,334	9,514	1,033	1,256	1,130	636	170	4,636	1,300	1,930	1,167	764	5,915	370	*29,451*
PBL(PHA)	*9,434*	*19,464*	*8,288*	*18,434*	*18,820*	*10,483*	*12,800*	*14,886*	*13,980*	*23,040*	*11,448*	*16,968*	*16,536*	*17,750*	*30,512*	*16,544*	*21,132*	*19,732*	ND	ND

Samples were taken from brain plaques of three patients with chronic progressive MS, and three controls (non-MS). Total RNA and cDNA (from 5 μg RNA) were prepared according to standard procedures[33]. Control cDNA was also prepared from 1 μg RNA isolated from a pool of peripheral blood lymphocytes from five different individuals, stimulated with 3 μg ml^{-1} of PHA. cDNAs were amplified with TCR Vα-Cα or actin primers for 40 cycles in the presence of 10 μCi of α-[^{32}P]dATP (Amersham). Samples were analysed by gel electrophoresis with ethidium bromide to identify the specific fragment band (italic figs when a band was clearly visible). After electrophoretic separation, bands were excised and incorporation of radiolabel was determined. Where TCR rearranged bands were absent, an agarose fragment 200–600 bp was excised and counted. An actin band was visualized in all the amplified brain cDNA. Results are expressed in counts per min. All TCR 5′ primers amplify TCR sequences from germ line DNA using a specific 3′ Vα primer for each family. We have detected V-Cα rearrangements of all TCR V gene members in a variety of activated T cells including single rearrangements of specific Vα members in T-cell clones reactive to pertussis toxin, to Borrelia bergdorfei, as well as the Jurkat T cell line, and rearrangements of all Vα members in pooled T cells stimulated by PHA (line 7, experiment 2). ND, Not determined.

on cDNA isolated from the parietal and occipital brain regions from one MS patient, and from the occipital brain region of one control (non-MS) individual, are shown in Fig. 1. Actin sequences were coamplified together with the Vα 12.1 gene to monitor the integrity of the cDNAs. Actin could be amplified from the brain cDNAs (500 base pair (bp) fragment (actin primer: lanes a–c) but not from PGA5, a full-length cDNA clone that contains the Vα 12.1 segment[18] (lane d). A smaller PCR product corresponding to the Vα 12.1 gene was detected in the patient but not in the control sample (282-bp fragment; primer AB13-AB14: lanes b, c and d). To ensure that only the Vα 12.1 family was amplified, genomic and brain cDNA PCR products were analysed using restriction endonucleases and were consistent with the known restriction map for Vα 12.1. When colonies containing cloned Vα PCR products from MS brain cDNA were screened with a Vα 12.1 region probe, about 20% were positive. DNA from several of these colonies was sequenced, and was found to be identical with the Vα 12.1 sequence[18]. Thus, the TCR Vα 12.1 restriction fragment-length polymorphism recently associated with MS susceptibility[15], must be in a sequence flanking the Vα 12.1 gene. Specific regulation of the α-TCR gene by 3′ *cis*-acting enhancers, was recently demonstrated[19].

These experiments indicated that PCR could amplify the receptor transcripts from post-mortem brain samples, starting from nanograms of total RNA without the necessity of *in vitro* expansion of T cells. Similarly, Cα sequences were amplified from MS brain cDNAs, but not from the control sample (primers AB41-AB42: lanes b and c, Fig. 1). A subsequent amplification was carried out (primers AB13-AB42: lanes a, b, c and d) using a 5′ primer complementary to the Vα 12.1 and a 3′ primer complementary to the Cα TCR regions. These primers, which can amplify only rearranged TCR transcripts, amplified a product of ~680 bp from both the positive control PGA5 sample and cDNA from the occipital region of the MS brain (primers AB13-AB42: lanes c and d) but not from control brain cDNA or cDNA from the parietal region of the MS brain (primers AB13-AB42: lanes a and b). The Vα and Cα amplifications from the MS brain parietal region most probably represent transcripts from unrearranged chromosomes, as has been found in other cDNA libraries from T cell lines[20]. No PCR product was observed using primers corresponding to the Vβ 8 family, even though this TCR Vβ region was recently reported to be associated with susceptibility to MS (ref. 13).

To substantiate that the DNA produced by PCR was an authentic amplified product of rearranged TCR genes, the PCR products were sequenced using the dideoxy chain termination method[21] after double screening of colonies with Vα and Cα probes. Only two different J regions were seen in the 25 clones examined. Both were different from the PGA5 Jα sequence, ruling out the possibility of a 'carryover' contamination. Eleven clones contained the JαO family found in clone HAP 41 (ref.

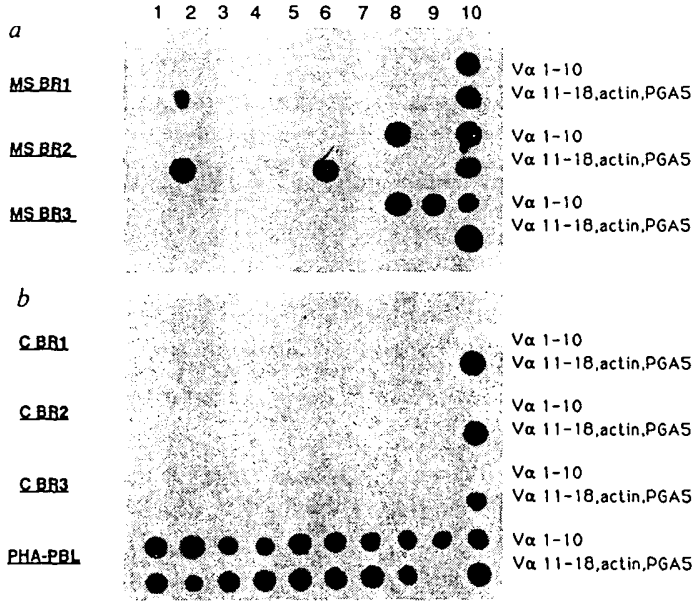

FIG. 2 Detection of PCR-amplified products by dot-blot hybridization using a Cα probe. *a*, MS brain (MS BR) amplifications; *b*, control brain (C BR) and PHA-PBL amplifications.
METHODS. The amplified product (10 μl) was dot-blotted onto transfer membranes (GeneScreen Plus, NEN) after denaturation with 0.4 M NaOH–25 mM Na_2EDTA. Actin PCR products served as negative controls and PGA5 as the positive control. Filters were fixed under ultraviolet light and prehybridized for 3 h at 42 °C in 5×SSPE/5× Denhardt's solution/salmon sperm DNA (100 μg ml^{-1})/0.1% SDS and hybridized overnight at 42 °C with 1 × 10^6 c.p.m. per ml of ^{32}P-kinased probe (5′-AATATCCAGAACCCTGACCCT-3′). Filters were washed in 1×SSPE/0.1% SDS at room temperature, twice for 20 min; and then in 0.1×SSPE/0.1% SDS at 45 °C twice for 10 min. Kodak XAR-5 film with Dupont Lightning-Plus intensity screens were used for autoradiography at −70 °C for 30 min. Signals correlate with the presence of a specific band in the ethidium bromide-stained agarose gel and with high [^{32}P]dATP incorporation during amplification (Table 2).

22) and 14 clones had a previously undescribed Jα sequence (GGGTACCGAGATGACGAACCCACCTTTGGGACAGG-CACTCAGCTAAAAGTGGAACTC).

We next asked whether there was a diverse or limited usage of TCR Vα gene expression in MS brain lesions. To analyse the use of TCR Vα in MS brains, we synthesized 5′ PCR primers for the 18 different Vα families[22,23] (Table 1). Optimal conditions for amplification were ascertained using each of the 5′ primers (Table 1) in combination with a specific 3′ Vα primer for each TCR Vα family, on genomic DNA (data not shown) and with a common Cα primer (AB 51) on reverse-transcribed RNA isolated from phytohaemagglutunin (PHA)-stimulated peripheral blood lymphocytes (Table 2). The results from amplification of MS brain cDNA, using 5′ Vα primers and the common 3′ Cα primer AB 51 in the presence of α-[^{32}P]dATP, showed that in each brain only a few TCR Vα gene families are preferentially rearranged and transcribed (Table 2). Visualization of a specific band for actin or rearranged TCR after gel electrophoresis of the amplified product, is indicated by italics in Table 2. In general, high [^{32}P]dATP incorporation was reproducibly detected in regions of the gel where bands were visualized.

These results were further confirmed by dot blot of the PCR products and hybridization with a radiolabelled oligonucleotide corresponding to a TCR-Cα sequence, 5′ from the common Cα primer (Fig. 2). A clear signal in the dot-blot assay correlates with the presence of a band staining with ethidium bromide in the agarose gel. Products of amplifications with actin primers were used as a negative control; amplification products of clone PGA5 with TCR Vα and Cα primers were used as a positive control. Expression of TCR Vα transcripts is operationally defined by (1) the presence of a rearranged Vα-Cα band visualized with ethidium bromide, and (2) a signal on dot-blot hybridization of the amplified Vα-Cα gene with a TCR Cα probe, 5′ from the Cα PCR primer. Thus in MS brain 1, TCR Vα 10 and 12 were rearranged; TCR Vα 8, 10, 12 and 16 were rearranged in MS brain 2, and Vα 8, 9 and 10 were rearranged in MS brain 3. No Vα transcripts could be amplified from control brain cDNA, although actin could be amplified. Each of 18 TCR Vα rearranged genes were amplified from PHA-stimulated pooled lymphocytes. The Vα 10 family was detected in all three MS samples, suggesting that this TCR might be responding to a major epitope of an antigen involved in the pathogenesis of MS. Further experiments will be needed to confirm this hypothesis.

Our results may prove to have therapeutic implications. Studies on TCR gene expression in experimental allergic encephalomyelitis (EAE), a prototypic animal model for autoimmunity induced by T cells, have shown the predominant use of certain TCR α and β products in the immune response to myelin basic protein[24–27]. Treatment of mice *in vivo* who have EAE with monoclonal antibody specific to the predominant TCR V-region product reverses paralytic disease[24]. In addition, it is possible to prevent EAE by immunization with inactive encephalitogenic T cells[28] or with synthetic peptides from the CDR2 and CDR3 TCR V regions that are rearranged in the encephalitogenic clones[29,30]. Elucidation of TCR expression in the brain may help in the design of similar treatments in MS patients. □

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Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter

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INHIBITORY non-adrenergic non-cholinergic (NANC) nerves are thought to be important in the autonomic innervation of the gastrointestinal tract and other organ systems. The nature of their neurotransmitter is still debated. Speculation that nitric oxide (NO), formed from L-arginine in neuronal structures[1] and other cells[2], could act as a neurotransmitter, is not yet supported by demonstration of its release upon nerve stimulation. Using a superfusion bioassay, we report the release of a vasorelaxant factor upon stimulation of the NANC nerves[3] in the canine ileocolonic junction. Several pieces of evidence, including the selectivity of the bioassay tissues, chemical instability, inactivation by superoxide anion and haemoglobin, inhibition by N^G-nitro-L-arginine (L-NNA)[4] and potentiation by L-arginine all indicated that NO accounted for the biological activity of this transferable NANC factor.

The canine ileocolonic junction (ICJ) was isolated, superfused with Krebs-Ringer solution and the effluent superfused de-endothelialized rings of rabbit aorta[5], arranged either in a cascade[6,7] (Fig. 1) or in parallel. Upon electrical stimulation (ES; 16 Hz, 2 ms) or infusion of the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP), ICJ tissue released a vasodilator activity, causing 22.4 ± 3.2% ($n = 12$) and 26.3 ± 3.9% ($n = 9$) relaxation of the top tissue respectively. The release of the factor was frequency-dependent and its activity declined (39 ± 8%, $n = 8$, ES; 41 ± 15%, $n = 4$, DMPP) during passage down the cascade. As atropine and guanethidine were present and as the detector tissues failed to relax to acetylcholine (ACh) and noradrenaline (Figs 1 and 2), the transferable factor is indeed non-adrenergic and non-cholinergic. Furthermore, blockade of nerve conductance with tetrodotoxin abolished the release of the vasodepressant factor to both stimuli (Fig. 2), indicating that neuronal structures were activated. Non-selective stimulation of other cell populations[2] or microorganisms therefore seems unlikely.

The instability of the vasodilator factor was compatible with NO[7,8], as confirmed by the injection of authentic NO (Fig. 1). Moreover, haemoglobin (Hb), known to trap NO avidly[7–9], eliminated the biological activity, whereas dilatation, due to NO

Contracted by 100nM noradrenaline

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